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Down-regulating Nrf2 by tangeretin reverses multiple drug resistance to both chemotherapy and EGFR tyrosine kinase inhibitors in lung cancer

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ABSTRACT

Multiple drug resistance (MDR) is the major obstacle for both chemotherapy and molecular-targeted therapy for cancer, which is mainly caused by overexpression of ABC transporters or genetic mutation of drug targets. Based on previous studies, we hypothesized that ROS/Nrf2 is the common target for overcoming acquired drug resistance to both targeted therapy and chemotherapy treatments. In this study, we firstly proved that the levels of ROS and Nrf2 were remarkably up-regulated in both H1975 (Gefitinib-resistant lung cancer cells with *T790M*) and A549/T (paclitaxel-resistant) cells, which is consistent with the clinical database analysis results of lung cancer patients that Nrf2 expression level is negatively related to survival rate. Nrf2 Knockdown with siRNA or tangeretin (TG, a flavonoid isolated from citrus peels) inhibited the MDR cell growth by suppressing the Nrf2 pathway, and efficiently enhanced the anti-tumor effects of paclitaxel and AZD9291 (the third generation of TKI) in A549/T or H1975, respectively. Moreover, TG sensitized A549/T cells-derived xenografts to paclitaxel via inhibiting Nrf2 and its downstream target P-gp, leading to an increased paclitaxel concentration in tumors. Collectively, targeting Nrf2 to enhance ROS may be a common target for overcoming the acquired drug resistance and enhancing the therapeutic effects of chemotherapy and molecular-targeted therapy.

1. Introduction

Lung cancer is one of the leading causes of mortality among cancer diseases, and non-small cell lung cancer (NSCLC) accounted for more than 85 % of patients with lung cancer. The first-line treatment is chemotherapy for NSCLC patients without apparent gene mutations or with unknown mutations. Recently targeted therapy such as tyrosine kinase inhibitor (TKIs) of the epidermal growth factor receptor (EGFR) targeting the EGFR mutation showed clinical benefits to special patients whose tumors harbor activating mutations within EFGR. However, acquired multidrug resistance (MDR) causes inefficacy of both chemotherapies and targeted therapy, leading to treatment failure [1]. Overcoming multidrug resistance is of profound meaning but still a challenge all over the world.

Overexpression of efflux transporters such as P-glycoprotein (P-gp) [2] is the most common molecular mechanism for MDR to chemotherapy. Several P-gp inhibitors have been developed as MDR sensitizers [3] and are currently under clinical phased studies [4]. However, there are limited therapeutic effects in clinical trials for MDR sensitizer due to the less selectivity to specific transporter [5].

Abbreviations: ABC, ATP-binding cassette; CI, combination index; DAPI, 4′6-diamino-2-phenylindole; DOX, doxorubicin; EGFR, Epidermal growth factor receptor; IC50, half maximal inhibitory concentrations; IHC, Immunohistochemistry; I.P., intraperitoneal injection; keap1, kelch-like ECH-associating protein 1; MDR, Multidrug resistance; MRP, multidrug resistance-associated protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NSCLC, non-small cell lung cancer; Nrf2, nuclear factor erythroid 2-related factor 2; P-gp, P-glycoprotein; PTX, paclitaxel; Rho 123, rhodamin123; tBHQ, tert-Butylhydroquinone; TKIs, tyrosine kinase inhibitors; UPLC-MS/MS, ultra-performance liquid chromatography-tandem mass spectrometry; Ver, verapamil.

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Although EGFR-TKIs such as gefitinib and erlotinib have been effectively used for clinical treatment of NSCLC, most patients developed resistance after 6–13 months TKIs treatment due to the development of secondary mutation (*EGFR* T790M) [6,7]. Recently, the third generation of EGFR inhibitors such as Osimertinib (AZD9291), rociletinib, and olmutinib, were developed for selectively binding to EGFR T790M mutation sites [8]. However, secondary resistance to third-generation inhibitors is now emerging in the literature [9], which may be related to further EGFR mutations and activation of other by-pass signaling pathways. As the underlying mechanisms are still unknown, efficient treatment for drug-resistance cancer is still a challenge to overcome.

Redox signaling is involved in physiological functions and the development of cancer. Redox balance plays an essential role in protecting human healthy and regulating cellular metabolism and proliferation [10,11]. As we described previously, gefitinib-resistant NSCLC cells exhibit an extremely high level of ROS [12]. Moreover, the cellular antioxidant signal Nrf2 has been demonstrated to promote the survival of cancer cells against oxidative stress, chemotherapeutic agents, and radiotherapy. Nrf2 is activated in response to the EGFR gene mutations in NSCLC cells [13]. Therefore, we hypothesized that ROS/Nrf2 is the common target for acquired drug resistance to both targeted therapy and chemotherapy treatments.

In this study, we investigated whether regulating redox balance via the Nrf2 pathway could be a common therapeutic targeting strategy to re-sensitize both efflux transporters overexpression and molecular targeted mutated drug resistance by using tangeretin (TG), a flavonoid isolated from citrus peels. In the previous study, we noticed that TG antagonized ABCB1-mediated MDR, but how it modulates the function of the transporter is remain unknown. Moreover, TG has been reported for its protective role in liver injury [14], renal injury [15], and arthritis [16] by activating Nrf2, while it is unclear the relationship between its effects on sensitizing MDR and Nrf2 pathway. EGFR double mutated lung cancer cell H1975 which are resistant to gefitinib (EGFR TKI) and paclitaxel (PTX) -resistant NSCLC cells A549T with P-gp overexpression were used as the typical MDR models for studying the reversal effects of tangeretin. Hopefully, the Nrf2 pathway inhibitor will be a novel strategy to overcome acquired drug resistance for both chemotherapy drugs and EGFR TKIs.

2. Materials and methods

2.1. Chemicals and reagents

Tangeretin ($C_{20}H_{20}O_7$, purity ≥ 98 % by HPLC detection), paclitaxel ($C_{47}H_{51}NO_{14}$, purity > 99 % by HPLC detection), verapamil (Ver, $C_{27}H_{38}N_2O_4$.HCl, purity > 98 % by HPLC detection), tertiary butylhydroquinone (tBHQ), and dimethylsulfoxide (DMSO) were purchased from Dalian Meilun Biology Technology Co., Ltd. The TG structure and purity were confirmed by LC-MS in our lab. RIPA lysis buffer (10 ×) and primary antibodies of GAPDH, Nrf2, P-gp were purchased from Cell Signaling Technology (Danvers, MA, United States). siRNAs targeting Nrf2 (si-Nrf2) were obtained from GenePharma (Shanghai, China). ROS probe, FBS, penicillin, streptomycin, Opti-MEM® I Reduced Serum Medium, Lipofectamine™ LTX Reagent, RNA extraction kit, Prolong® Gold Anti-fade Reagent with DAPI were bought from Invitrogen (Carlsbad, CA, United States). Taq DNA Polymerase Kit and cDNA synthesis kit were purchased from Roche (Basel, Switzerland). The plasmid extraction kit was purchased from Favogene (Ping-Tung, Taiwan).

2.2. Cell lines and cell culture

Human non-small cell lung cancer (NSCLC) cell lines NCI-H1819, A549, NCI-H1975, HCC827, and HCT-8 cell lines were purchased from ATCC. ABCB1-overexpressing drug-resistant cell line A549/T was purchased from KeyGen Biotech Co., Ltd. (Nanjing, China). All cell lines have been authenticated using Short Tandem Repeat (STR) DNA profiling. Cells were cultivated with RPMI 1640 medium supplemented with 10 % FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (GIBCO, Paisley, Scotland) at 37 °C with a humidified atmosphere of 5 % CO_2. To maintain drug resistance, A549/T cells were continuous exposure to 0.24 μM paclitaxel.

2.3. MTT assay

Cell viability was assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) Assay. Briefly, 5000 cells/ well were seeded on a 96-well microplate and cultured overnight for cell adhesion. After treatment, MTT (5 mg/ml, 10 μl) solution was added to every well and incubated for 4 h. After solubilization of the formazan crystals, the colorimetric signal of the plate was measured at 570 nm (absorbance) and 650 nm (reference) using a microplate reader. The cell viability was calculated as the percentage change of the absorbance of the treated cells divided by the absorbance of the untreated cells.

2.4. Reactive oxygen species detection

The cellular ROS levels were determined by ROS Assay Kit using a cell-permeant fluorogenic dye 2′,7′-dichlorofluorescein diacetate (DCFDA). The cells were pre-treated with DCFDA at a working concentration of 20 μM for 30 min at 37 °C. Then, the cells were incubated with test compounds or vehicle control for a specific duration followed by harvesting. The ROS level was measured with a flow cytometer with excitation and emission settings of 488 and 525 nm, respectively.

2.5. Assessment of apoptosis levels via annexin V/PI staining

After being harvested, cells were washed with PBS twice and resuspended in the binding buffer. Then, the cells were stained with Annexin V-FITC and propidium iodide (PI) for 15 min at room temperature in the dark. The apoptotic cells were quantitatively counted with an Aria III flow cytometer.

2.6. Immunofluorescence microscopy

Cells grow on glass coverslips and culture overnight for cell adhesion. After treatment, the cells were fixed with 1 ml of 4 % PFA for 15 min and then washed with PBS 3 times. Triton X-100 (1 ml of 0.1 %) was added to the cells and incubated for 5 min to penetrate the cell membrane. Cells were incubated with the primary antibody in blocking solution (1:500 dilution) at 4 °C overnight and washed 3 times with PBS. Then, the cells were incubated in a fluorochrome-conjugated secondary antibody that was diluted in the blocking solution (1:500) for 1 h at room temperature in the dark. Finally, cover slides were washed and fixed with Prolong® Gold Anti-fade Reagent with DAPI. The immunofluorescence images were captured with DeltaVision Live Cell Imaging System.

2.7. Animal Xenograft model

All animal studies were approved by the Animal Care and Use Committee at Guangzhou University of Chinese Medicine (No #ZYYL20150807). To build up the Xenograft model, A549/T cells were injected subcutaneously at the flank near the armpits of four-week-old female BALB/c nude mice. After tumor size approximately reached 100 mm3 (day 0), mice were randomized into four groups (n = 6 per group): vehicle (control); PTX (15 mg/kg); TG (50 mg/kg) [17,18]; PTX with TG (15 mg/kg PTX and 50 mg/kg TG). The treatments were given via intraperitoneal injection (I.P.) every 3 days for a total of 9 doses. The tumor growth was monitored every 3 days and was calculated using the following equation: volume = ($width^2$ × length)/2. At the end of the experiment, the blood and tumor were collected and immediately

weight before storing at − 80 °C for further analysis.

2.8. Immunohistochemical (IHC) analysis

To examine the expression of Nrf2 in the tumor tissue samples, 4-μm-thick sections of tumors, which were fixed with 4 % paraformaldehyde, and permeabilized with 0.1 % Triton X-300 in PBS containing 5 % bovine serum albumin, were used for immunohistochemistry (IHC) analysis. The sections were incubated for 2 h with the primary antibody (0.5 μg/ml Nrf2 monoclonal antibody), and then incubated with diluted biotinylated goat anti-mouse antibody. Strept Avidin HRP and then Vector VIP Substrate (Vector Laboratories, Burlingame, CA) were used for staining as well as 4′6-diamino-2-phenylindole (DAPI). The image was captured by an inverted fluorescence microscope.

2.9. Western blotting

The protein concentration of cells lysate was determined by Bio-Rad DCTM Protein Assay Kit. Equal amounts of cell lysates (40 μg) were loaded and electrophoresed onto a 10–12 % SDS-PAGE gel. The separated proteins were transferred to a nitrocellulose (NC) membrane. After blocking with 5 % milk without fat in tris-buffered saline containing 0.1 % of Tween20 (TBST) for 1 hr at room temperature, primary antibodies (1:1000 dilutions) were incubated overnight at 4 °C. After washing the membrane three times with TBST (5 min/wash), secondary fluorescent antibody (1:10,000 dilutions) was added to the membrane at room temperature and incubated for 1 hr. The signal intensity of the target proteins was detected by LI-COR Odyssey Scanner.

2.10. UPLC-MS/MS analysis of paclitaxel

Sample preparation and LC-MS determination were carried out according to our previously reported method [19]. The concentration of PTX in tumor fractions was analyzed with an Agilent 6460 Triple Quadrupole LC/MS System (Agilent Technologies, Inc., USA) using a Waters ACQUITY UPLC BEH C18 column (1.7 μm 2.1 × 50 mm; Waters, Torrance, CA). A linear gradient mobile phase which composed of 0.1 % formic acid water (A) and 0.1 % formic acid in methanol (B) was optimized according to the following gradient program:0–5 min (30 % B); 5–7 min (30–55 % B);7–10 min (55–85 % B); 10–11 min (85–30 % B). The injection volume was 5 μl at 4 °C and the flow rate was 0.4 ml/min. Electrospray (ESI) source was used in positive ion mode. The source parameters were set as follows: capillary voltage, + 4000 V; fragment or voltage, 150 V; drying gas, 11 L/min; drying gas temperature, 325 °C; nebulizer gas pressure, 45 psi; and dwell time, 200 ms. M/Z for PTX is 876.32/308.10 (CE 24 V), M/Z for docetaxel (Internal standard) is

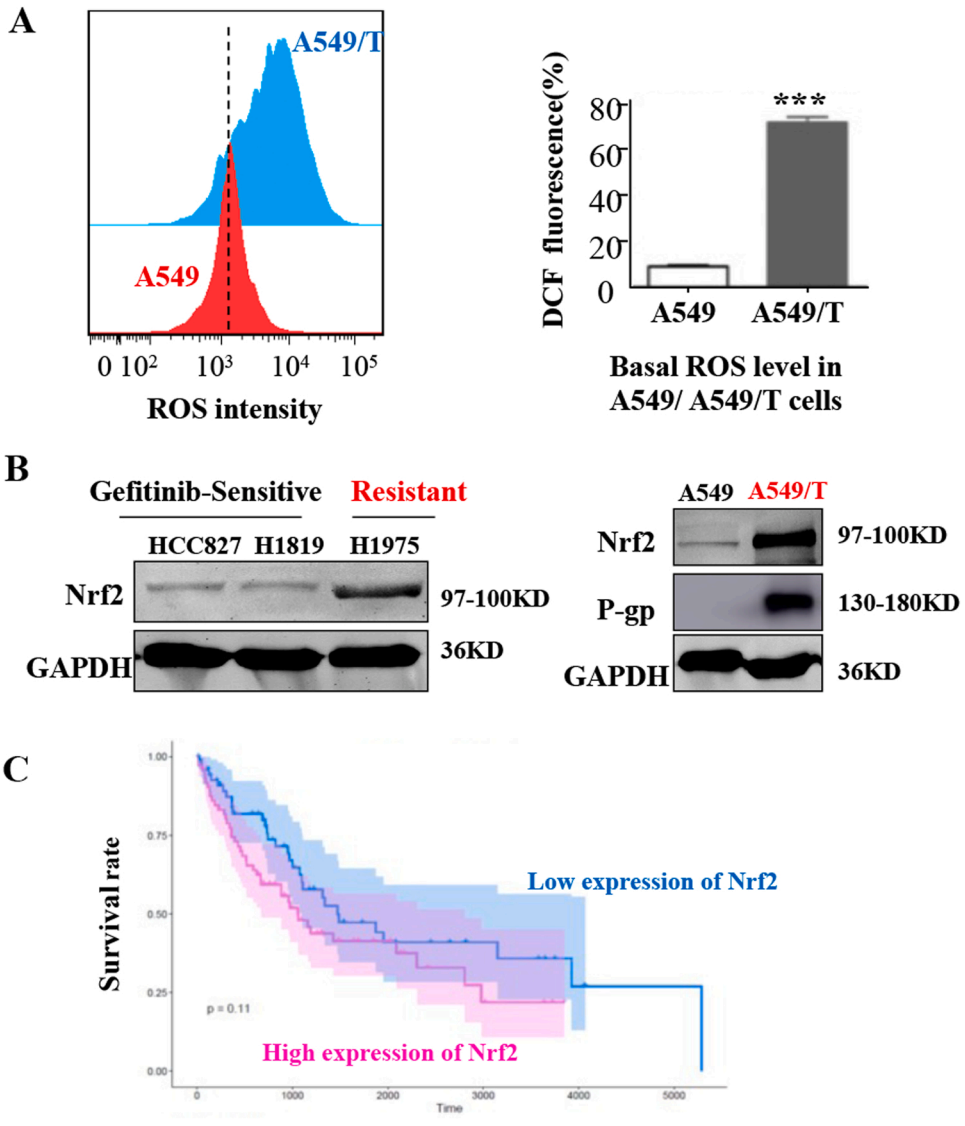

Fig. 1. ROS production as well as the expression of Nrf2 in the gefitinib sensitive/resistant (sensitive cell H1819, HCC827, and resistant H1975) and paclitaxel sensitive/resistant (A549 and A549/T) cancer cells. A) The basal ROS level increased in resistant cells in comparison with sensitive parental A549 cells; B) The protein level of Nrf2 in two types of resistant lung cancer cells: gefitinib sensitive/resistant (H1819, HCC827, H1975) and paclitaxel sensitive/resistant (A549, A549/T); C) The patient survival rate is negatively related with the expression of Nrf2 based on the Kaplan Meier survival analysis of the Cancer Genome Atlas (TCGA) database. Data were presented as the mean ± SD ($n = 4$). ***$P < 0.001$ vs. A549 group.

830.34/549.20 (CE 24 V). All UPLC–MS/MS data were obtained by Agilent ChemStation Software (v6.03).

2.11. Statistical analysis

All data were expressed as the means ± SEM of three independent experiments. Statistical analysis was determined using the one-way analysis of variance (ANOVA) or Student's t-test with GraphPad Prism 5, which was followed by Bonferroni's test to compare all pairs of columns. The level of significance was set at $P < 0.05$ (*), < 0.01 (**) or < 0.001(***) for all tests.

3. Results

3.1. Reactive oxygen species (ROS) and Nrf2 were remarkably elevated in drug-resistant NSCLC

Compared with sensitive A549 lung cancer cells, ROS was up-regulated in drug-resistant A549/T cells (Fig. 1A). Similarly, the EGFR TKI-resistant H1975 cells exhibited an extremely high ROS level than sensitive cancer cells in our previous report [12]. It is interesting that both resistant NSCLC cell models contained higher basal ROS levels than sensitive cancer cells.

Excessive ROS level is detrimental to cells. So, we assumed that scavenging ability should be enhanced as well in resistant cancer cells to remove the increased intercellular ROS. As the key regulator of anti-oxidation, the expression of Nrf2 in resistant cancer cells was compared with sensitive cells. As shown in Fig. 1B, consistent with ROS results, Nrf2 was remarkably increased in both drug-resistant NSCLC cells A549/T and H1975. The increased levels of ROS and antioxidant ability indicated by the expression of Nrf2 are the common characteristics of resistant lung cancer cells.

Furthermore, Kaplan Meier's survival analysis of the Cancer Genome Atlas (TCGA) database of lung cancer patients suggested that Nrf2 expression level is negatively related to survival rate (Fig. 1C), indicating that overexpression of Nrf2 protected tumor cells from cell death and was associated with poorer therapeutic effects.

Therefore, these results suggested that overexpression of Nrf2 may be one of the common targets for overcoming drug resistance in NSCLC cancer cells.

3.2. NRF2 knockdown inhibited the proliferation of resistant NSCLC cells

To explore whether Nrf2 is essential for the proliferation of drug-resistant of lung cancer cells, siRNA was applied to knock down Nrf2. As shown in Figs. 2A, 2D and 2G, knockdown of Nrf2 significantly induced apoptosis and inhibited the growth of resistant A549/T cells. But knockdown of Nrf2 had only a minimal inhibition effect on the sensitive parental A549 cells (Figs. 2B, 2E and 2H).

Similar results were obtained in H1975 which has been demonstrated to have a high level of Nrf2. Nrf2 knockdown blocked the growth of H1975 cells (Fig. 2C and F) as well as its phenotype (Fig. 2I). This discrimination between resistant and sensitive cancer cells suggested that regulating ROS balance conferred selectively cytotoxic to drug-resistant cancer cells.

Moreover, we testified whether activation of Nrf2 could induce the

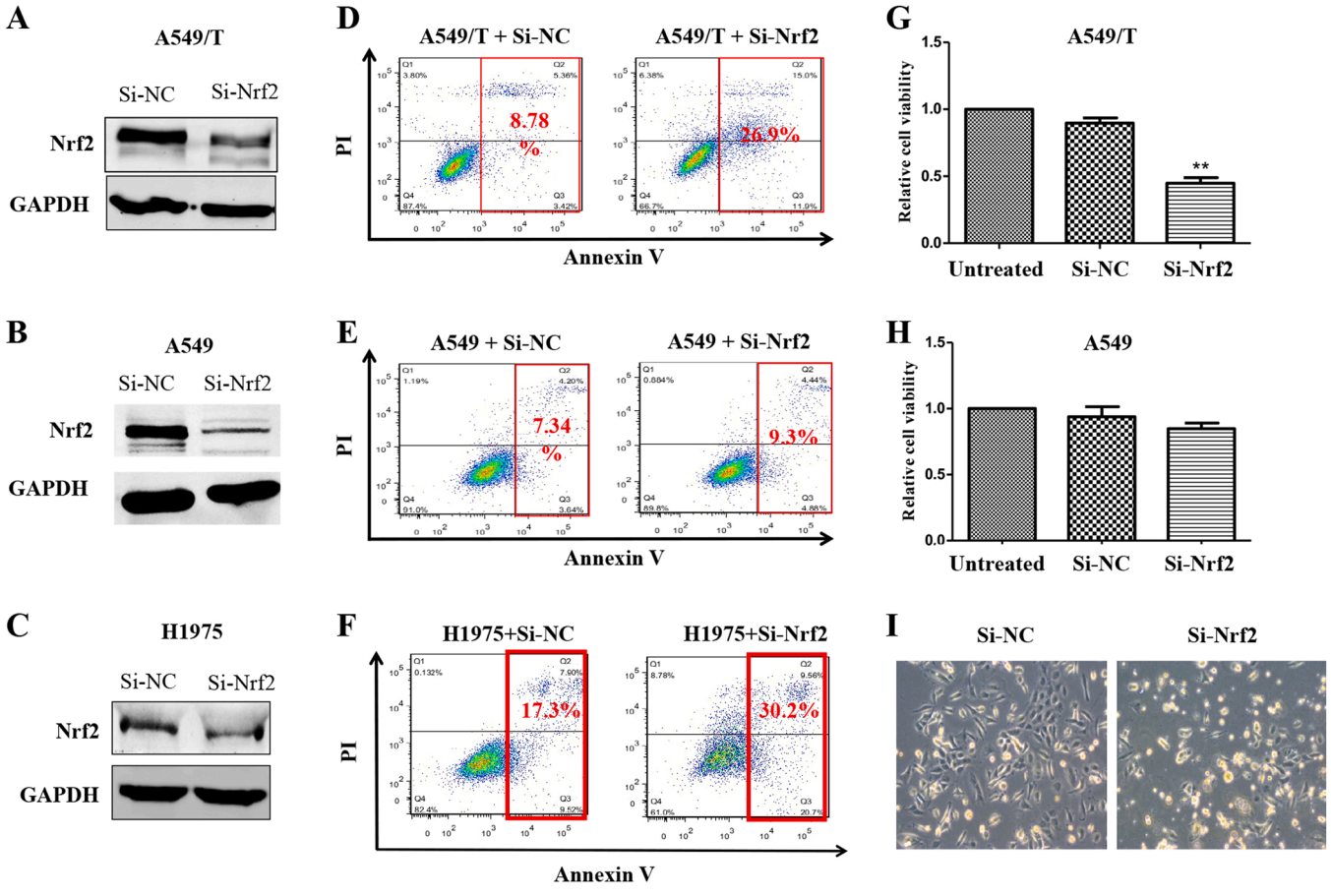

Fig. 2. Knockdown of Nrf2 via siRNA plays anticancer activity. The markable apoptotic effect of siRNA-Nrf2 (A) on A549/T drug-resistant cancer cells (D and G), but not in the A549 cancer cells (B, E and H). Similarly, knockdown of Nrf2 via siRNA (C) induced the apoptosis of gefitinib resistant H1975 (F) as well as altered cell morphology images (I, original magnification × 200). Cell viability was assessed by MTT Assay. Data were presented as the mean ± SD ($n = 3$). **$P < 0.01$ vs. control group.

resistance of cancer cells to the treatment of PTX. In the sensitive parental A549 cells, the anti-cancer efficacy of PTX was mostly abrogated after adding the Nrf2 activator tBHQ (Fig. 3A and B). Taken together, these results demonstrated that Nrf2 upregulation may be the potential target for overcoming the drug-resistant induced by PTX and EGFR-TKIs in lung cancer cells.

3.3. Tangeretin overcomes the drug resistance via suppressing Nrf2

To find an effective natural Nrf2 inhibitor, we screened our compounds library to identify the potential compounds. Tangeretin (TG), a flavonoid isolated from citrus peels, was selected based on the inhibition effect on Nrf2 and low toxicity in our primary study [17]. Both immunofluorescence (Fig. 4A) and western-blot (Fig. 4B) results presented the suppressive effect of TG on the overexpression of Nrf2, which is corresponding to the IC50 of TG in A549/T cells.

Importantly, the co-treatment of TG could reverse the resistance to PTX (0.94 μM) and remarkably enhance the cytotoxic effect of PTX on A549/T cells by inhibiting the expression of Nrf2 as shown in Fig. 5 A and 5B. The percentage of apoptotic cells increased from 7.6% to 51.0 % after co-treatment of TG and PTX compared with PTX alone.

Similarly, the activation of Nrf2 was suppressed by TG in H1975 cells which have a mutation in exon 20 (T790M) in a dose-dependent manner (Fig. 6A and B). TG at 20 μM significantly increased the percentage of apoptotic cells to 23.2 %. In contrast, AZD9291 (the third generation of EGFR TKI) at 4 μM only has a weak cytotoxic effect on H1975 cells by comparing with TG, indicating that TG may have a stronger antitumor effect on EGFR mutation NSCLC. Here, we also find the markable synergetic effect of TG with AZD9291 in H1975 cells. As shown in Fig. 6C, co-treatment of TG with AZD9291 lead to about 59.8 % of apoptotic cells.

Therefore, down-regulation of Nrf2 by TG overcame the resistance to A549/T and H1975 cells, and increased the cytotoxic effects of PTX and AZD9291 in the resistant lung cancer cells.

3.4. TG increased the intracellular accumulation of drugs via suppressing Nrf2/P-gp

Nrf2 plays a critical role in transcriptional upregulation of the expression of ABC transporter genes in resistant cancer cells [20–22]. To study the Nrf2-inhibition effects and anti-MDR mechanism of TG, we investigated the expression of P-gp in A549/T cells in the absence or presence of TG or Nrf2 si-RNA. As shown in Fig. 7A, knockdown of Nrf2 via siRNA remarkably downregulated the expression of P-gp. Similarly, TG suppressed the expression of P-gp via inhibiting Nrf2 in a dose-dependent manner.

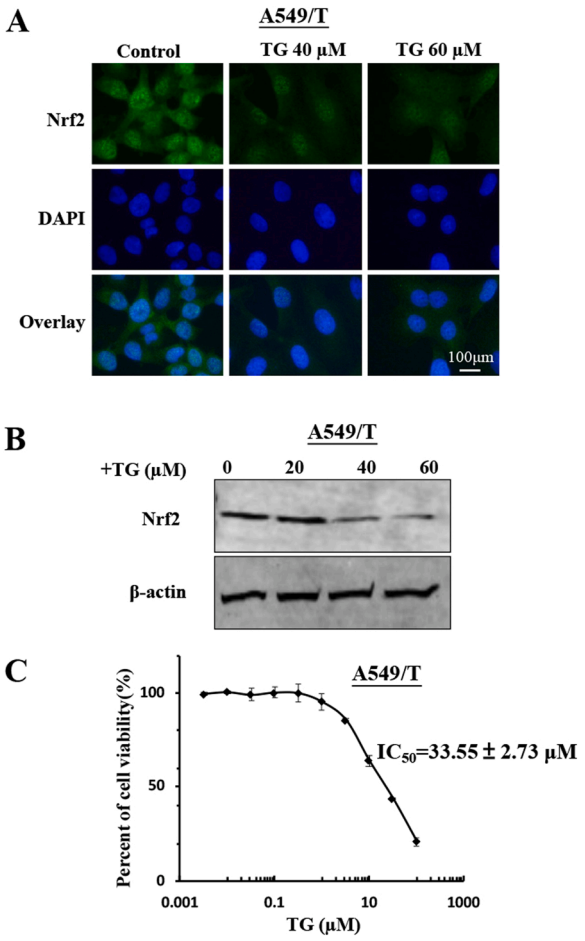

Fig. 4. Inhibition of Nrf2 by tangeretin (TG) induced apoptosis in drug-resistant A549/T cancer cells. TG suppressed the Nrf2 levels in A549/T cells with A) fluorescence macroscopy images and B) Western-Blot analysis. C) The IC_{50} value of TG, which is consistent with the dosage for suppressing Nrf2. Data were presented as the mean ± SD ($n = 3$).

P-gp overexpression, inducing the reflux of drugs, results in a low intercellular concentration, which is the most classic mechanism for the acquired resistance to chemotherapy in many cancers [23]. In Fig. 7C, the intracellular level of Rho123 which is the substrate of P-gp [24] was

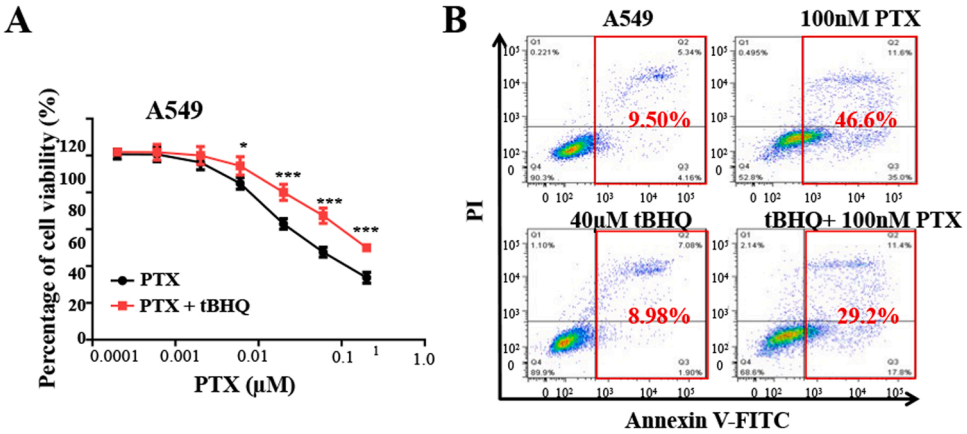

Fig. 3. Activation of Nrf2 by tBHQ leads to drug resistance in A549 cells. A) The IC_{50} value of PTX in the presence or absence of tBHQ (40 μM) in A549 cells; B) Treatment of tBHQ (40 μM) partially blocked the cytotoxic effect of PTX in A549 cells. Data were presented as the mean ± SD ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. PTX alone group.

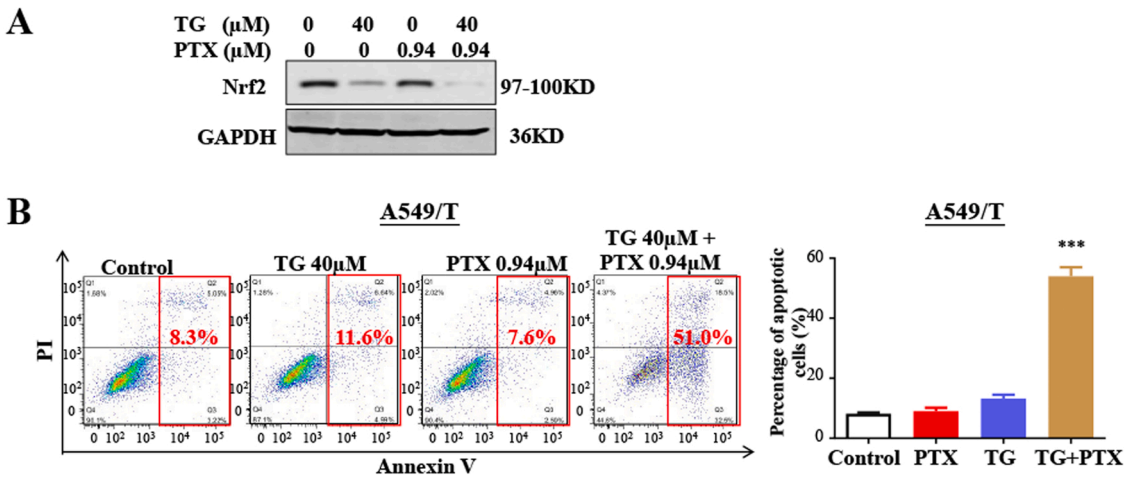

Fig. 5. Tangeretin (TG) increased the cytotoxic effects of paclitaxel in resistant A549/T cells. A) TG alone (40 μM) or in combination with PTX (0.94 μM) significantly inhibited the expression of Nrf2.; and B) TG (40 μM) remarkably enhanced the cytotoxic effect of PTX (0.94 μM) on A549/T cells. Data were presented as the mean ± SD ($n = 4$). ***$P < 0.001$ vs. PTX alone group.

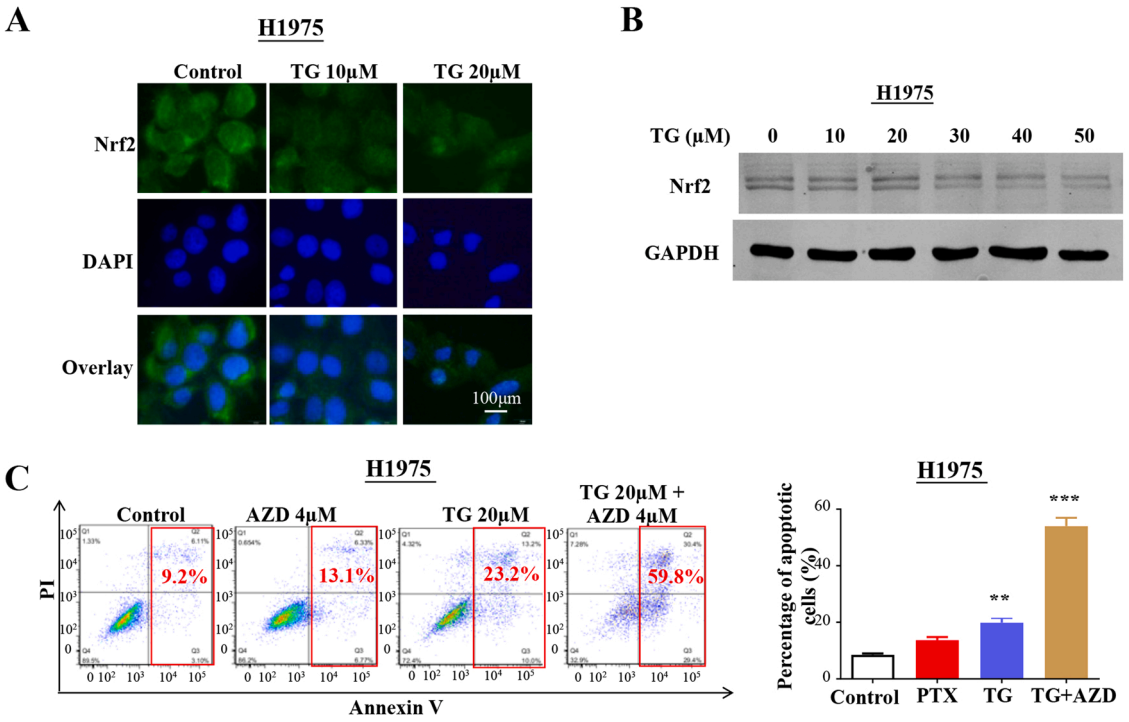

Fig. 6. Tangeretin not only induced the apoptosis of H1975 cells, but also increased the cytotoxic effects of AZD9291(the third generation of EGFR-TKI) in gefitinib-resistant H1975 cells. The expression of Nrf2 was suppressed by TG in H1975 cells in a dose-dependent manner as shown in A) fluorescence macroscopy images and B) Western-Blot analysis. C) Tangeretin at 20 μM induced the apoptosis of H1975 cells which is stronger than the effect of AZD9291 at 4 μM, while co-treatment of TG and AZD9291 showed synergistic anti-tumor effects in H1975 cells. Data were presented as the mean ± SD ($n = 3$). **$P < 0.01$, vs. control group; ***$P < 0.001$ vs. AZD alone group.

low in A549/T under immunofluorescence microscopy due to the overexpression of P-gp. Here, both TG and verapamil increased the intracellular accumulation of Rho123 in A549/T.

3.5. TG sensitized A549/T cells-derived xenografts to PTX via inhibiting Nrf2

To demonstrate the anti-MDR effect of TG in vivo, an NSCLC PTX-resistant A549/T xenograft mouse model was applied in BALB/c-nu/nu mice. As shown in Fig. 8, the tumors showed fast growth in the vehicle-treated group and TG group. The dose of PTX at 15 mg/kg which had shown therapeutic effects in several experiments [25,26] was used. PTX at 15 mg/kg did not affect the body weight of mice, indicating a well-tolerated dose of PTX. As expected, PTX alone did not slow the tumor growth.

However, as shown in Fig. 8 A and B, TG sensitized drug-resistant tumors to PTX and remarkably enhanced the anti-cancer effects of PTX. Both tumor volume and weight were strikingly shrieked in the combination group. Moreover, TG could markedly decrease the protein level of Nrf2 in A549/T cells-derived xenografts as well as in the combination group (Fig. 8D).

LC-MS detection also substantiated the inhibiting effect of TG on the

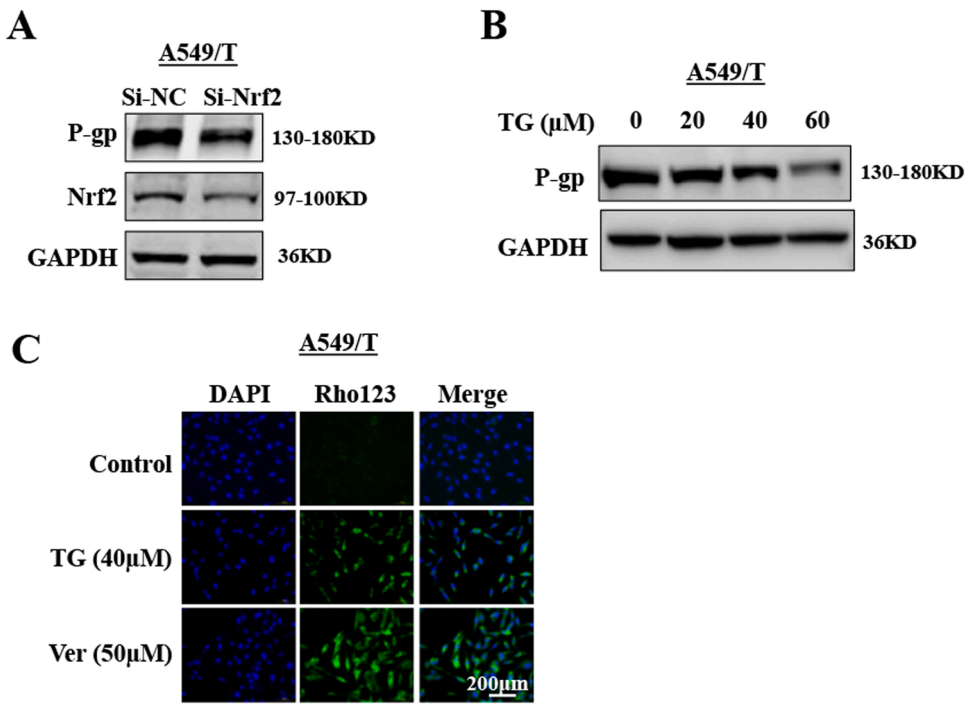

Fig. 7. TG suppressed the Nrf2/P-gp pathways. A) knockdown of Nrf2 via siRNA reduced the expression of P-gp; B) TG also inhibited the expression of P-gp. C) TG enhanced the accumulation of Rho123, the substrate of P-gp in A549/T cells. Verapamil (VER), the inhibitor of P-gp, was used as the positive control. Data were presented as the mean ± SD ($n = 3$).

overexpression of Nrf2/P-gp in the tumors of A549/T xenografts. Compared to the PTX given alone group, co-treatment of TG increased the tumor concentration of PTX approximately 3-fold (Fig. 8E).

Therefore, suppression of Nrf2 could sensitize the drug-resistant NSCLC cells to chemotherapy and TKIs in vitro and in vivo, while TG as an Nrf2 inhibitor has the potential as a novel MDR sensitizer.

4. Discussion

Today, despite the new chemotherapy agents or target therapy medicines growing, drug resistance continues to be a serious problem in the field of cancer, which is related to 90% of failures in the chemotherapy [27]. The acquired resistance in both chemotherapy and targeted therapies can be major caused by two mechanisms which are the alterations in drug transport and/or metabolism, mutation, and/or amplification of drug targets. However, what is the common mechanism leads to the alteration of drug transporter and secondary mutation? In the previous studies [12,19], we noticed a common feature in both acquired drug resistance to chemotherapy and EFGR-TKIs. Therefore, for the first time as we know, we hypothesized that ROS/Nrf2 may be the common target for overcoming drug resistance. In this study, we demonstrated that the surge of ROS and up-regulation of Nrf2 are commonly observed in two different drug-resistant NSCLC with efflux transporters overexpression or secondary EGRF mutation, which could be the target for different kind of drug resistance acquired in cancer treatment.

Generally, ROS enhances apoptosis and autophagy for non-MDR cancer cells [28]. However, our previous work has identified that the basal ROS levels in EGFR(T790M)-containing TKI-resistant NSCLC cell lines were substantially high compared to non-MDR cancer and normal cells [12,29] The role of ROS in developing resistance to anti-cancer drugs has been well-proposed: i) elevated ROS exhausts cellular reducing agents, like glutathione which serves as a cofactor in facilitating MDR protein-mediated drug efflux and thus conferred resistance to anti-cancer drug toxicity [30]; ii) ROS could induce genetic instability and lead to further DNA mutations [31,32]. In response to the excess of intercellular ROS which may lead to cell death, resistance cancer cells had increased Nrf2 to against the electrophiles and oxidants and enhanced the survival of cells, which may be the reason for the "apoptosis evasion" of MDR cancer cells. Therefore, Nrf2 possessed great potential as a target for breaking the balance of oxidative stress and antioxidant defense within drug-resistant cancer cells, leading to cell apoptosis. Here, from in vitro and in vivo studies, we demonstrated that regulating ROS through inhibiting Nrf2 is effective and promising for anti-drug resistance in different cancer therapies.

Moreover, Nrf2, a pivotal transcriptional factor, is well known for regulating the expression of efflux transporters, such as MRP family members [33]. Recently, a positive correlation between Nrf2 and P-gp was observed in both colorectal cancer [34] and gastric cancer patients [35]. However, few studies demonstrated the direct regulation of P-gp by Nrf2 in lung cancer. In this study, we found that knock-down or suppression of Nrf2 could reduce the P-gp level, which helps to explain the role of ROS in modulating efflux transporters in drug-resistant cancer cells. Consistently, inhibition of Nrf2 enhanced the efficacy of anti-cancer drugs by accumulating its intercellular concentrations in drug-resistant tumors.

TG, a flavone in tangerine peels, showed multiple pharmacological effects such as antioxidative and anti-inflammatory. It promoted the expression of Nrf2 and inhibited the expression of KEAP1, which contributes to its protective role in liver injury [14], renal injury[15], and arthritis [16]. Nrf2 activators showed beneficial effects on many chronic diseases that are underlined by oxidative stress and inflammation [36]. However, Nrf2 is overexpressed in many tumors and contributes to resistance to chemotherapy and TKIs. Here, we found that TG was an inhibitor of Nrf2 in drug-resistant cancer cells. In the literature, natural compounds such as luteolin [37] and wogonin [38] were reported to inhibit Nrf2, but they also elicited Nrf2 activation [39]. Keap1, a sensor for the degradation of Nrf2 protein, has many highly reactive thiols which are the targets of oxidative stresses or compounds. Moreover, the substrate adapter component of the E3 ligase (Keap1-Cul3 complex) and the thiol groups of Keap1 are directly modified by the stimuli, as a unique feature of Keap1-Nrf2 pathway [40,41]. All these characteristics

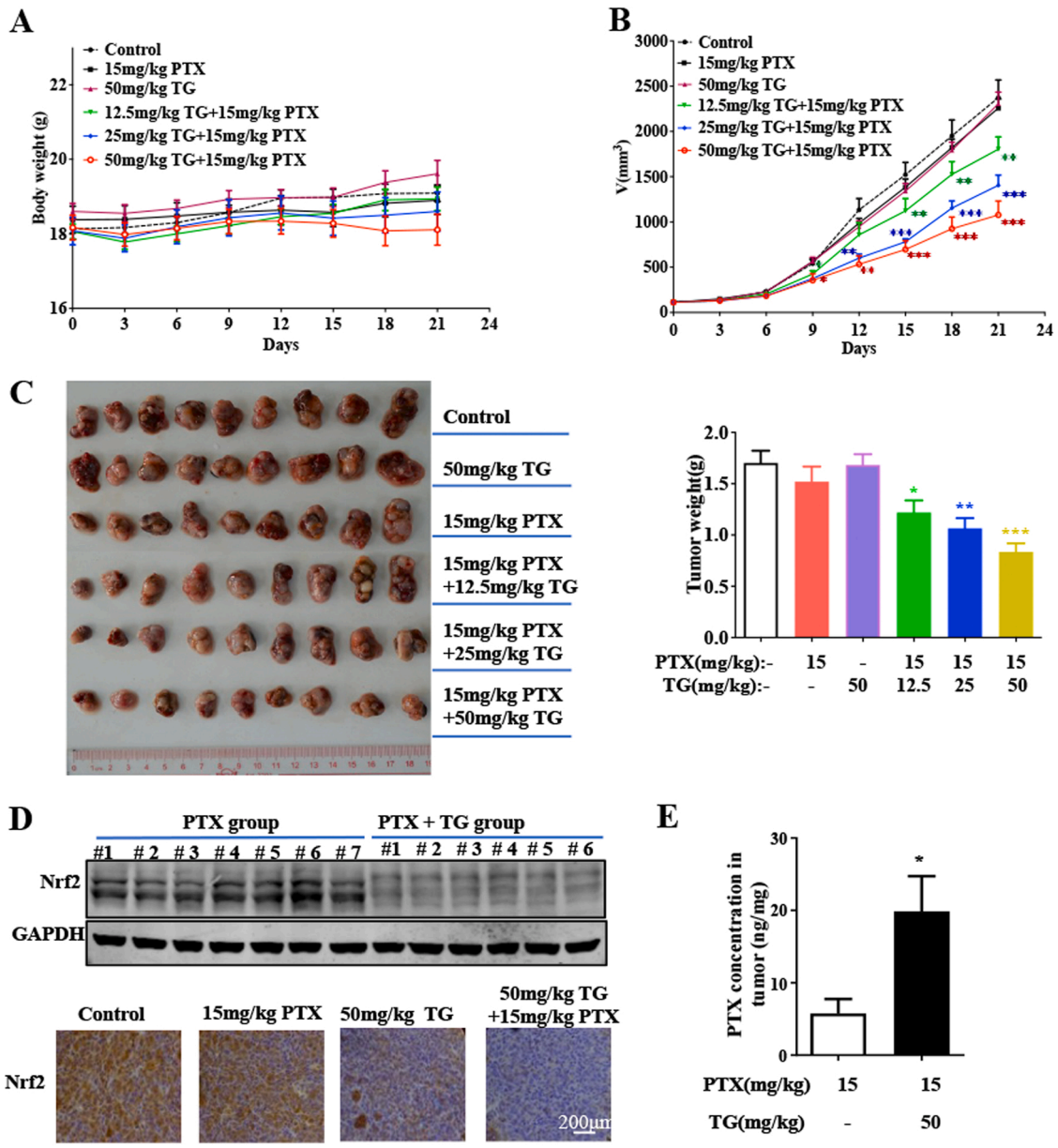

Fig. 8. Tangeretin (TG) increased the anti-cancer therapeutic effects of paclitaxel (PTX) in vivo in A549/T xenograft model. Co-treatment of TG (50 mg/kg) with PTX significantly reduced the tumor size (A), tumor volume (B) and tumor weight (C), and delayed the growth of the tumor, while PTX (15 mg/kg) alone have no anticancer effects ($n = 9$). D) IHC and Western-Blot analysis showed that TG remarkably down-regulated the Nrf2 level in mouse tumor tissues; F) TG significantly enhanced the concentrations of PTX in the xenograft tumors. Data were presented as the mean ± SD ($n = 9$). *$P < 0.05$, vs. PTX alone group.

make the response of Keap1–Nrf2 pathway varied in different diseases, and also provide different targets for modulating the keap1-Nrf2 interaction by natural compounds.

Therefore, Nrf2 inhibitor TG and si-RNA have the great potential to be developed as a combination therapy for use with PTX or EFGR-TKIs.

CRediT authorship contribution statement

Conceptualization and study design: Ying Xie, Xing-Xing Fan, Hua Zhou; Methodology: Ying Xie, Elaine Lai-Han Leung; acquisition of data: Sen-ling Feng, Fang He, Xing-Xing Fan; analysis and interpretation of data: Ying Xie, Pei-Yu Yan, Xiao-Jun Yao, Hua Zhou; Writing – review & editing, and/or revision of the manuscript: Ying Xie, Xing-Xing Fan; Funding acquisition: Ying Xie, Xing-Xing Fan. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

All authors declared there are no conflicts of interest.

Data Availability

Data will be made available on request.

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